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14. ABSTRACT We hypothesize that ER stress induced by glucose in diabetes promotes diabetic CKD through CRT stimulation of TGF-beta-dependent calcium/NFAT signaling in renal proximal tubule cells. In Aim 1 we will determine the role of CRT in mediating the fibrogenic effects of TGF-beta and glucose in renal cells. In Aim 2, we will determine the role of CRT in mouse models of diabetic nephropathy. To date, we have established cultures of mouse proximal tubule cells (MPTC) isolated from CRT-floxed mice and shown that TGF-beta and glucose stimulate ECM (fibronectin, collagen) in both MPTC and in human PT cells (HK-2). In HK-2 cells, we showed that siRNA for CRT reduces TGF-beta stimulation of ECM. Cre-recombinase plasmid reduced CRT expression in MPTC. We are developing lentiviral mouse and human CRT shRNA approaches to establish stably transduced cell lines and for use in long-term assays. For aim 2, we established that 0.7 mPa of ultrasound to deliver cre-recombinase plasmid to the kidney is safe (no urinary AKI markers) and reduces CRT protein in renal tubules by 80-65% over days 3-14 by IHC and blot of protein lysate. CRT is expressed primarily in the tubules. These results suggest the feasibility of the proposed studies.					
15. SUBJECT TERMS Diabetes, chronic kidney disease, diabetic nephropathy, TGF-beta, calreticulin					
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1. INTRODUCTION

Rationale: ER stress promotes the development of diabetic nephropathy. The diabetic milieu increases ER stress through hyperglycemia, advanced glycation end products, oxidative stress, and decreased ER Ca^{2+} stores. While ER stress is initially adaptive, chronic stimulation leads to cell death and fibrosis. There is emerging evidence linking ER stress with fibrosis, but mechanisms are unclear. In diabetic CKD, transforming growth factor-beta (TGF-beta) stimulation of extracellular matrix (ECM) is central to development of diabetic CKD. We recently identified the ER stress protein, calreticulin (CRT), as a critical regulator of TGF-beta stimulation of ECM, thereby establishing a mechanistic link between ER stress and fibrosis. CRT is an ER chaperone and a regulator of ER calcium homeostasis and of nuclear factor of activated T cells (NFAT) signaling. High glucose and TGF-beta increase CRT in cultured proximal tubule cells. Increased CRT is observed in mouse models of tubulointerstitial fibrosis and in diabetic nephropathy. We showed that *CRT is required for TGF-beta transcriptional stimulation of ECM* and that CRT deficient cells fail to respond to TGF-beta ECM stimulation. CRT knockdown in human proximal tubule (HK-2) cells attenuates TGF-beta and high glucose stimulation of fibronectin and collagen I. CRT overexpression increases responses to TGF-beta. CRT mediates TGF-beta signaling through regulation of TGF-beta-stimulated calcium release and stimulation of NFAT activity. High glucose increases NFAT activity in HK-2 cells and NFAT blockade reduces diabetic renal fibrosis. These data provide evidence that CRT is critical for TGF-beta and glucose stimulated ECM production, suggesting a new mechanism by which CRT drives ER stress-dependent diabetic CKD. We hypothesize that *ER stress induced by glucose in diabetes promotes diabetic CKD through CRT stimulation of TGF-beta-dependent calcium/NFAT signaling in renal proximal tubule cells.* This will be tested in the following aims:

Specific Aim 1: To determine the role of CRT in mediating the fibrogenic effects of TGF-beta and glucose in renal cells Tubular injury and fibrosis are major determinants of progression to CKD. We will address whether CRT and/or NFAT regulate TGF-beta and glucose stimulation of the fibrotic phenotype in HK-2 cells by assessing ECM production, mesenchymal phenotype, migration, and resistance to apoptosis. We will use HK-2 cells subjected to CRT siRNA knockdown and proximal tubule cells isolated from CRT-floxed mice transduced with lentiviral Cre-recombinase. We will use NFAT inhibitors to address which responses are NFAT-dependent. Diabetes is an oxidative environment and hydrogen peroxide (H_2O_2), a mediator of oxidant signaling, can regulate TGF-beta dependent calcium signaling and ECM stimulation. Thus, we will address the potential role of CRT in regulating calcium release downstream of glucose/TGF-beta and H_2O_2 .

Specific Aim 2: To determine the role of CRT in mouse models of diabetic nephropathy We will use several approaches to downregulate CRT to determine the role of CRT in diabetic CKD: we expect that CRT downregulation will attenuate renal fibrosis and improve renal function. **Aim 2a:** We will knockdown proximal tubule CRT in streptozotocin-treated CRT floxed B6D2F1 mice using a proximal tubule promoter-specific (PEPCK) cre-recombinase plasmid delivered using kidney targeted microbubble/ultrasound-mediated plasmid delivery. We will also examine non-targeted CRT knockdown in these mice. **Aim 2.b:** We will similarly drive CRT knockdown using CRT RNAi plasmid delivery in the Akita/C57Bl6 uninephrectomy model of type 1 diabetes in which fibrosis is primarily tubulointerstitial. **Aim 2.c:** We will directly assess the role of NFAT in diabetic CKD through use of the NFAT inhibitor, 11R-VIVIT, in the Akita model. Both gene delivery and disease analyses will be performed with the assistance of the NIH-funded UAB O'Brien Center.

2. KEYWORDS: diabetes, chronic kidney disease, diabetic nephropathy, calreticulin, TGF-beta, ER stress, ultrasound, tubulointerstitial fibrosis

3. ACCOMPLISHMENTS

a. WHAT WERE THE MAJOR GOALS OF THE PROJECT?

Tasks for year 1 (months 1-12) are highlighted in bold

Research-Specific Tasks:

Specific Aim 1: To determine the role of CRT in mediating the fibrogenic effects of TGF-β and glucose in renal cells	Months	Progress towards completion
Major Task 1: (Aim 1.1.a, 1.1b) Establish cell models of CRT knockdown in HK-2 and mouse proximal tubule cells	1-6	
Subtask 1: Confirm Not human subjects research for use of HK-2 cells through UAB IACUC and DoD HRPO Cell lines used: HK-2 [ATCC CRL-2190]	1-3	100%
Subtask 2: Isolate and culture mouse proximal tubule cells from CRT floxed mice	1-3	100%
Subtask 3: Validate CRT knockdown using human siRNA Cell lines used: HK-2 [ATCC CRL-2190]	3-6	100%
Subtask 4: Validate Cre Recombinase plasmid activity and CRT downregulation in transfected mouse proximal tubule cells	3-6	90%
<i>Milestone(s) Achieved: Obtain IRB/HRPO approval. Validation of CRT knockdown protocols in human and mouse cells</i>		
Major Task 2: (Aim 1.2) TGF-β and glucose response assays	7-20	Murphy-Ullrich
Subtask 1: measure ECM production in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	7-9	80% in HK-2 10% in mouse PTEC
Subtask 2: measure apoptosis in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	10-12	0%
Subtask 3: measure cell migration/invasion in response to TGF-beta and glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	12-18	Post-doc
Subtask 4: epithelial plasticity in response to TGF-beta and glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	19-20	Post-doc
<i>Milestone(s) Achieved: Determine role of CRT in tubular epithelial behavior in response to TGF-beta or glucose treatment</i>		
Major Task 3: (Aim 1.3) Role of CRT in TGF-β and glucose mediated calcium release and NFAT activity	21-25	Murphy-Ullrich
Subtask 1: Measure intracellular calcium release in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	21-23	Post-doc
Subtask 2: Measure NFAT activity (nuclear localization, reporter assay) in response to TGF-beta or glucose with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	24-25	Post-doc
<i>Milestone(s) Achieved: Determine role of CRT in calcium and NFAT regulation downstream of glucose and TGF-beta</i>		

Major Task 4: (Aim 1.4) Role of NFAT in CRT-regulated HK-2 responses	26-36	Murphy-Ullrich
Subtask 1: Use NAFT inhibitor any cellular responses determined from Major task 2 to be CRT-dependent Cells used: HK-2, CRT floxed mouse tubule cells	26-36	Post-doc
<i>Milestone(s) Achieved: determination of role of NFAT in cellular responses regulated by CRT</i>		
Major Task 5: (Aim 1.5) Role of CRT in regulating hydrogen peroxide-dependent increases in calcium release and NFAT activity	26-36	Murphy-Ullrich
Subtask 1: determine if catalase blocks TGF-beta and glucose stimulation of epithelial plasticity, ECM expression, calcium release, and NFAT reporter activity in cells with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	26-28	Post-doc
Subtask 2: measure hydrogen peroxide levels in TGF-beta and glucose stimulated cells +/- Smad inhibitor in cells with CRT knockdown Cells used: HK-2, CRT floxed mouse tubule cells	29-36	Post-doc
<i>Milestone(s) Achieved: Delineation of pathways by which CRT regulates cellular responses to glucose and TGF-beta; publication of 1-2 peer reviewed papers</i>		
Specific Aim 2: To determine the role of CRT in mouse models of diabetic nephropathy	Months	GSU
Major Task 1: (Aim 2.1) Drive proximal tubule specific and general Cre-recombinase expression in diabetic CRT-floxed mice (116 mice + breeders)	1-24	Murphy-Ullrich/Sanders
Subtask 1: Submit animal use request to UAB IACUC and DoD ACURO	1-2	100%
Subtask 2: Confirm sequence of PEPCK promoter plasmid and clone into CRE-recombinase GFP construct, validate in floxed mouse PT cells in vitro	1-2	0%
Subtask 3: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice	3-5	0%
Subtask 4: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (66 mice)	7-12	0%
Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	12-14	Murphy-Ullrich, Ailing Lu
Subtask 6: validate CRE-recombinase GFP construct in floxed mouse PT cells in vitro	12	80%
Subtask 7: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice	13-14	100%
Subtask 8: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (50 mice)	15-21	10%
Subtask 9: animal model analysis (histology, ECM, serum	21-24	Murphy-

creatinine, urinary albumin/creatinine, IHC for fibrosis markers)		Ullrich, Ailing Lu
<i>Milestone(s) Achieved: obtain IACUC/ ACURO approval, validation of model and ultrasound/microbubble plasmid delivery; determination of role of CRT on diabetic CKD; submission of manuscript</i>		
Major Task 2: (Aim 2.b) CRT shRNA knockdown in the Akita model of diabetic CKD (50 mice)	10-24	Murphy-Ullrich/Sanders
Subtask 1: identify active mouse CRT siRNA; develop shRNA plasmid under U6 promoter, and validate CRT knockdown with mouse cells (MEFS, mouse proximal tubule cells) in vitro.	10-12	50%
Subtask 2: Validate CRT shRNA knockdown delivered via renal artery in pilot study (5 mice)	12-14	Dr. Sanders/Core B, post-doc
Subtask 3: Perform uninephrectomy; confirm diabetes in Akita mouse (45 mice)	15	Core B/Ailing Lu
Subtask 4: Deliver CRT shRNA via renal artery; repeat as necessary	16-20	Core B/Ailing Lu
Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	21-24	Murphy-Ullrich, Ailing Lu, Core B
<i>Milestone(s) Achieved: validation of CRT shRNA approach and determination of role of CRT in the Akita uninephrectomy model; submission of manuscript combined with Major Task 1 of Aim 2.</i>		
Major Task 3: (Aim 2c) Determine the role of NFAT in diabetic nephropathy through use of a specific NFAT inhibitor (35 mice)	25-36	Murphy-Ullrich
Subtask 1: Perform uninephrectomy in Akita mouse; confirm diabetes	25	Sanders/Core B
Subtask 2: Deliver 11R-VIVIT peptide 3 x/week i.p. over 15 weeks	26-29	Ailing Lu
Subtask 3: : animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)	30-36	Murphy-Ullrich, Ailing Lu, Core B
<i>Milestone(s) Achieved: Determination of role of NFAT in tubulointerstitial fibrosis and renal function in the Akita uninephrectomy model; publish manuscript</i>		

b. WHAT AS ACCOMPLISHED UNDER THESE GOALS

Milestones for Year 1 (months 1-12)

Specific Aim 1: to determine the role of CRT in mediating the fibrogenic effects of TGF- β and glucose in renal cells

Major Task 1 (Aim 1.1a, 1.1b)

Subtask 1: Confirm Not human subjects research for use of HK-2 cells through UAB IACUC and DoD HRPO
Cell lines used: HK-2 [ATCC CRL-2190]

Completed

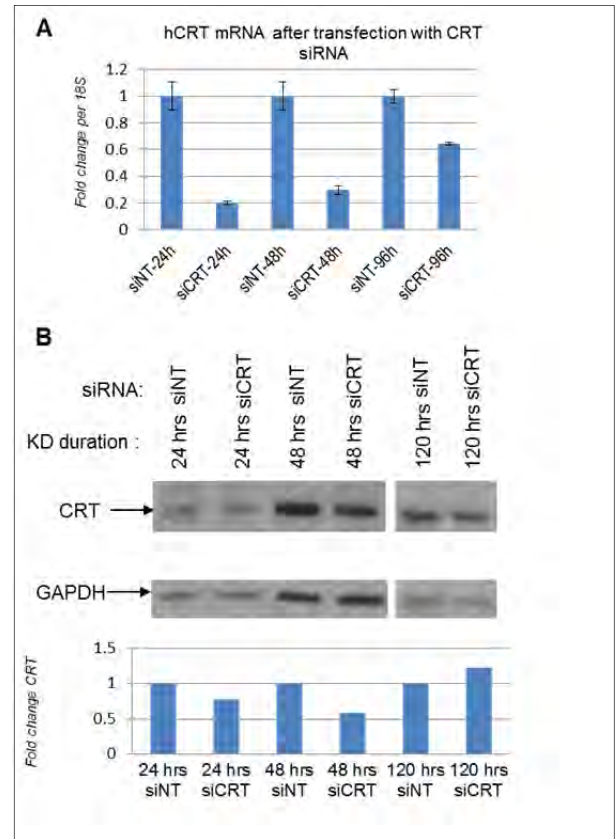
Subtask 2: Isolate and culture mouse proximal tubule cells from CRT floxed mice

Completed, we now can routinely culture these cells

Subtask 3: Validate CRT knockdown using human siRNA Cell lines used: HK-2 [ATCC CRL-2190]

Using electroporation, we showed that we can knockdown CRT mRNA (A) and protein (B) over a 96 hour period by delivering siRNA (**Figure 1**). In these studies and in others not shown, maximum down regulation of CRT protein occurred between 48-96 hrs post siRNA transfection with typically 40-60% knockdown, which has been sufficient to see effects in previous studies with fibroblasts and vascular smooth muscle cells.

Figure 1: Knockdown of CRT message and protein in HK-2 cells by siRNA over time HK-2 cells were transfected with siRNA to CRT or with non-targeting siRNA (NT). (A) Message levels for CRT were measured by RTQ-PCR and (B) protein by western blotting with densitometric analysis following normalization to GAPDH.



Subtask 4: Validate Cre Recombinase plasmid activity and CRT downregulation in transfected mouse proximal tubule cells

We have been able to culture multiple isolates of primary mouse proximal tubular epithelial (PT) cells. As these are primary cells, transfection efficiency has been low (30-40%). We used both lipofectamine and electroporation approaches with similar results. Despite the low transfection efficiency, we were able to detect nearly 60% downregulation of CRT protein in mouse cells transfected with Cre-recombinase plasmid, but not with GFP control plasmid (**Figure 2**). We also observed downregulation of CRT expression in vivo in mouse proximal tubules (see Major Task 2, Subtask 7). However, the in vitro transfection results were not entirely consistent (some transfections showed lower levels of CRT k.d.) and for experimental purposes, we will switch to use of a lentivirus to transduce mouse PT cells with cre-recombinase plasmid using a viral construct that contains an antibiotic resistance selection marker. This will yield stably transduced cells with a higher efficiency of CRT knockdown for in vitro studies.

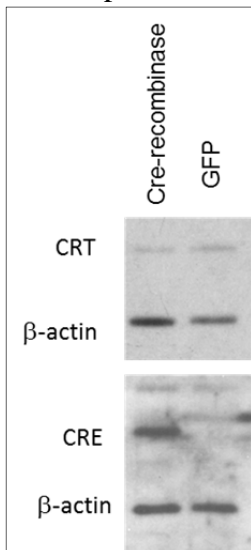


Figure 2: Knockdown of CRT protein in primary mouse proximal tubule cells isolated from CRT floxed mice. Mouse proximal tubule cells were transfected with plasmids expressing Cre-recombinase-IRES-GFP (CRE) or with plasmid expressing GFP. After 72 hrs, cells lysates were analyzed for CRT protein and also for CRE to validate expression of the plasmid. Densitometric analysis showed a 60% reduction in CRT protein.

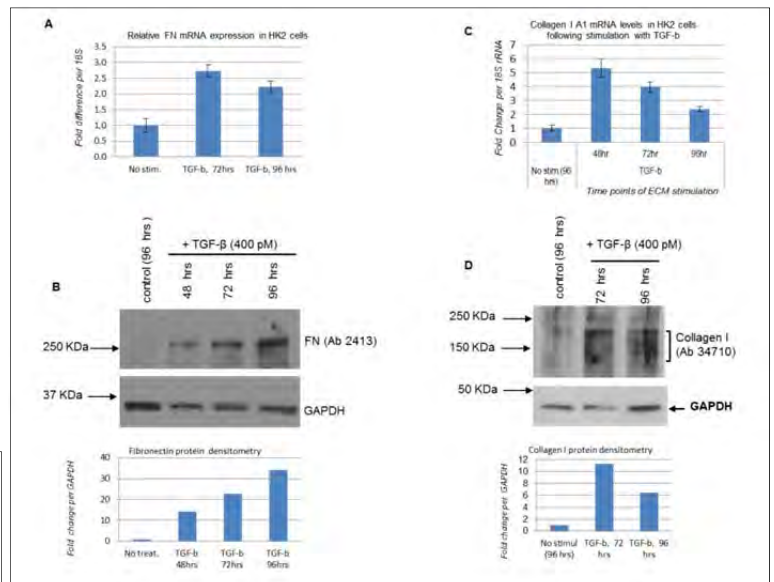
Major Task 2 (aim 1.2)

Subtask 1: measure ECM production in response to TGF-beta or glucose with CRT knockdown

Cells used: HK-2, CRT floxed mouse tubule cells

Studies showed that TGF- β stimulated fibronectin (FN) and type I collagen production in HK-2 cells

Figure 3: TGF- β stimulates fibronectin (FN) and collagen type I (col I) mRNA and protein expression by HK-2 cells.



at both the mRNA and protein levels (**Figure 3**). Similarly, TGF- β stimulated FN and col I mRNA and protein expression by mouse PT cells (**Figure 4**).

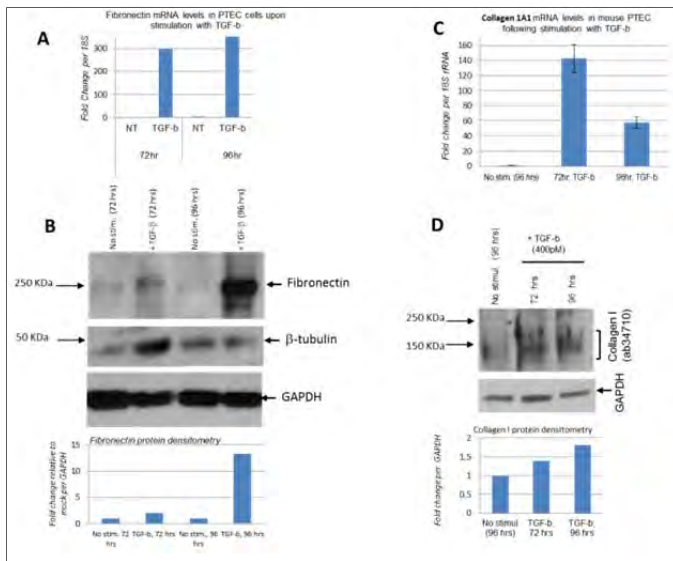


Figure 4: TGF- β stimulates fibronectin (FN) and collagen type I (col I) mRNA and protein expression by mouse proximal tubules cells isolated from CRT floxed mice.

In further studies, we treated HK-2 cells and mouse PT cells with either 5.5-6 or 30 mM glucose and examined FN and type I collagen production. FN was increased by 30 mM glucose relative to 5.5 mM glucose (Mouse PTC) or 6 mM glucose (HK-2) [designated as no stimulation condition]. Glucose stimulated FN expression in HK-2 cells at 72-96 hrs, although glucose induction of FN was more transient in the mouse PTC cells, peaking at 72 hrs. Collagen I protein, but not message, was also stimulated

by 30 mM glucose treatment of HK-2 cells at 72-96 hrs (**Figures 5, 6**).

Figure 5: Glucose (30 mM) stimulates FN and col I expression by HK-2 cells. HK-2 cells were grown in media with 6 mM glucose (control), 10 mM, or 30 mM glucose. 30 mM glucose stimulated increased FN mRNA and protein levels after 72-96 hrs of treatment. Col I protein was increased by 72 hrs with 30 mM glucose, although mRNA levels were not increased at this time point.

To determine the role of CRT in mediating TGF- β and glucose-dependent ECM production, HK-2 cells were stimulated with TGF- β following knockdown of CRT with siRNA (**Figure 7**) and collagen protein levels examined. CRT knockdown reduced TGF- β stimulated levels of collagen I protein. In year 2, we will repeat these studies to address the effects of CRT knockdown on TGF- β and glucose stimulation of FN and col I. These studies will also be repeated in the mouse PT cells.

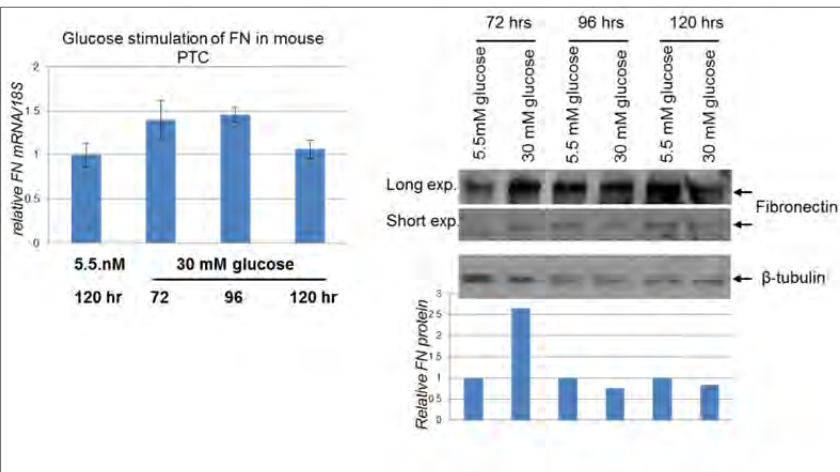
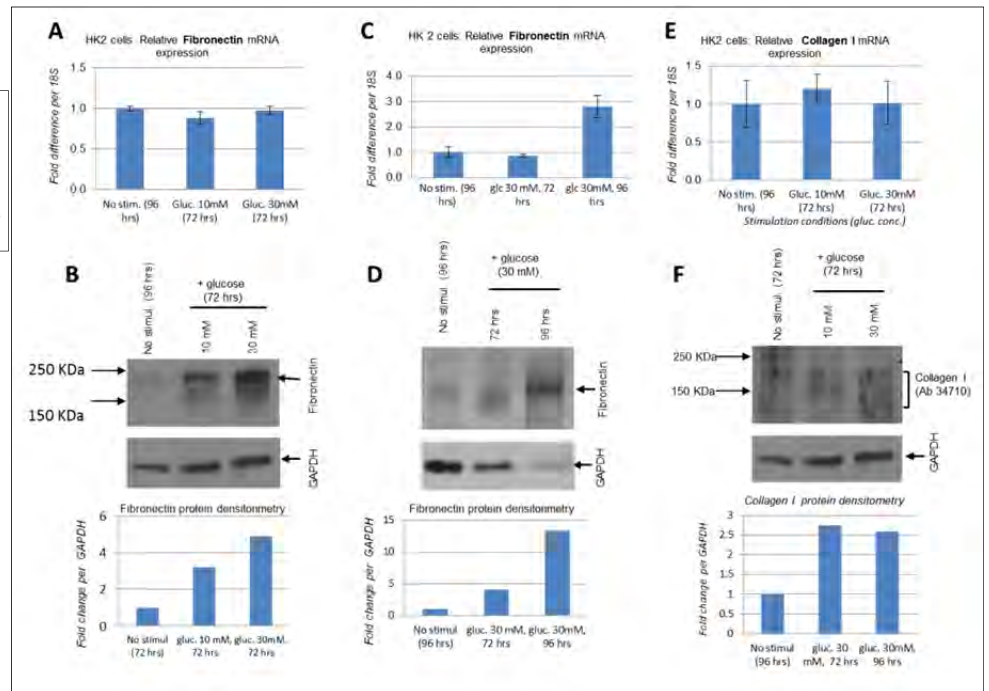


Figure 6: Glucose (30 mM) stimulates FN mRNA and protein expression by primary mouse PT cells. Mouse PT cells were grown in media with 5.5 mM glucose (non-stimulated control) or 30 mM glucose. 30 mM glucose stimulated increased FN mRNA and protein levels after 72 hrs of treatment.

Due to the variable transfection efficiencies, we have not been able to obtain reliable data with the mouse PT cells. We have identified several shRNA which are effective in mouse PT cells (see Major Task 2 (Aim 2.b, Subtask 1)). We expect that our lentiviral transduction approach to express CRT shRNA and selection of cells with stable CRT

knockdown will increase the feasibility of these studies.

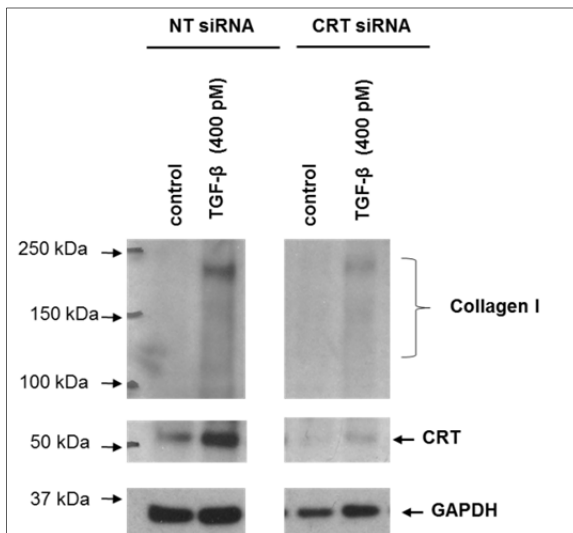


Figure 7: siRNA knockdown of CRT in HK-2 cells attenuates TGF- β stimulated col I protein expression. HK-2 cells were transfected with CRT or NT siRNA and 24 hrs later cells were stimulated with 400 pM TGF- β for 72 hr. Type I collagen levels were detected by western blotting of cell lysates. Blots also confirmed CRT knockdown.

Subtask 2: measure apoptosis in response to TGF-beta or glucose with CRT knockdown; Cells used: HK-2, CRT floxed mouse tubule cells

We did not make any progress on this task in year 1. Having the stably transduced clones will increase the success of this aim in year 2.

Specific Aim 2: to determine the role of CRT in mouse models of diabetic nephropathy

Major Task 1 Aim 2.1

Subtask 1: Submit animal use request to UAB IACUC and DoD ACURO

Completed. We have obtained UAB IACUC and DoD ACURO approval.

Subtask 2: Confirm sequence of PEPCK promoter plasmid and clone into CRE-recombinase GFP construct, validate in floxed mouse PT cells in vitro

Subtask 3: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice

Subtask 4: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (66 mice)

Subtask 5: animal model analysis (histology, ECM, serum creatinine, urinary albumin/creatinine, IHC for fibrosis markers)

Subtasks 2-5: These tasks are related to the PEPCK promoter plasmid and confirmation of renal delivery have been delayed. We are choosing to proceed with the general knockdown approach first, because there is little glomerular expression of CRT. Most CRT expression is tubular, although the IHC suggests collecting duct and distal tubules express CRT as well as proximal tubules. We will generate the PEPCK promoter driven Cre-recombinase in year 2.

Subtask 6: validate CRE-recombinase GFP construct in floxed mouse PT cells in vitro

These data are discussed under Major Task 1, subtask 4.

Subtask 7: Confirm plasmid delivery to renal tubules through ultrasound/microbubble sonoporation; Confirm CRT knockdown in tubules by PCR and protein in B6D2F1 CRT floxed mice

We performed two different pilot studies to assess the feasibility of using the ultrasound/microbubble (US/MB) approach to deliver cre-recombinase plasmid to the kidneys of floxed mice. Although we had

previously established the efficacy of this approach in a mouse carotid artery ligation model (Zimmerman et al, manuscript in revision), we had not yet established efficacy and toxicity in the kidney.

In the first pilot study, we treated mice (n=2-3/group) with 3 different intensities of US and delivered microbubbles without plasmid. Mice were injected with 200 μ l MB, rapidly anesthetized via isofluorane inhalation, and then the dorsal kidney areas were subjected to 0.2, 0.7, or 1.3 MPa of ultrasound pressure for a total of 1 min exposure, consisting of two 30 sec pulses with a 30 sec interval. Mice were harvested on days 2, 7, and 14 after MB/US treatment and 24 hr urines collected prior to sacrifice. Urines were analyzed for early (kim-1) and later markers of renal injury (albumin, creatinine). Hematoxylin & Eosin-stained kidney sections were also examined for signs of morphologic damage. Histologic analyses showed some tubular damage at the outer cortical regions of the kidney, especially in 1.3

MPa treated mice, and to a lower extent in the reduced US dosed mice (0.2, 0.7) (data not shown). However, the urinary analyses did not show elevations in kim-1, a marker of acute kidney injury, or in urinary albumin or creatinine (**Figure 8**).

In the second pilot study, we evaluated the efficacy of MB/US-mediated targeted cre-recombinase plasmid delivery to the kidney. The cre-recombinase plasmid was shown to have activity in reducing CRT expression of mouse PT cells isolated from the CRT floxed mice (Major task 1, Subtask 4, Figure 2). We used 3 animals/group. Mice were 8-17 weeks of age and of both sexes. Mice were treated via tail vein injection with 300 μ g of plasmid in 50 μ l of saline, which was combined with 200 μ l of Optison MBs as described for pilot study 1. Plasmids were either GFP or Cre-recombinase-IRES-GFP and were validated in our previous mouse carotid studies and in the mouse PT cells. Anesthetized animals were subjected to either 0.7 or 1.3 MPa of ultrasound as described in pilot study 1. Kidneys were harvested from animals at days 2, 7, and 14 following MB/US treatment/plasmid delivery.

Kidneys were analyzed for CRT mRNA by RT-PCR (1.3 MPa dose only), CRT protein in renal lysates by western blotting, and staining for CRT in kidney sections by immunohistochemistry and morphometric analyses. Interestingly, the higher dose US treatment (1.3 MPa) did not significantly knockdown CRT expression at either the mRNA or protein level in the kidneys (data not shown). However, we did observe downregulation of CRT protein in renal lysates and by IHC morphometric analyses in the kidneys from mice treated with 0.7 MPa of US (**Figures 9A-C**). Surprisingly, CRT protein knockdown occurred fairly rapidly (by 2 days), but persisted over 14 days. Note that CRT expression is localized primarily to the tubules and that MB/US delivery of cre-recombinase downregulates CRT expression in the tubules. We interpreted the response with 0.7 MPa US vs 1.3 MPa US to reflect a balance between US-mediated induction of CRT (due to cellular stress and/or mechanotransduction) and the ability of cre-recombinase to excise the CRT gene to reduce protein expression. Because of the predominance of CRT expression in the tubules, we have decided to proceed directly with animal studies without first generating the tubule specific PEPCK promoter constructs. Note that we are able to use tail vein delivery instead of direct delivery through the renal artery, which eliminates a surgical procedure.

ELISA for kim-1						
Urine	Ear tag number	days	24h Urine μ l	d2 pg/ml AVERAGE	d7 pg/ml AVERAGE	
Control	914	d7	765		2955.73	
No ultrasound	917	d7	1000		2757.37	
Group1 0.2 MPa	903	d2	540	1185.72		
	913	d7	530		4344.06	
	924	d7	650		2893.09	
Group2 0.7MPa	898	d2	315	6598.23		
	912	d7	119		3884.80	
Group3 1.3MPa	896	d2	410	7636.46		
	904	d7	355		3081.01	
	905	d7	1210		1331.96	

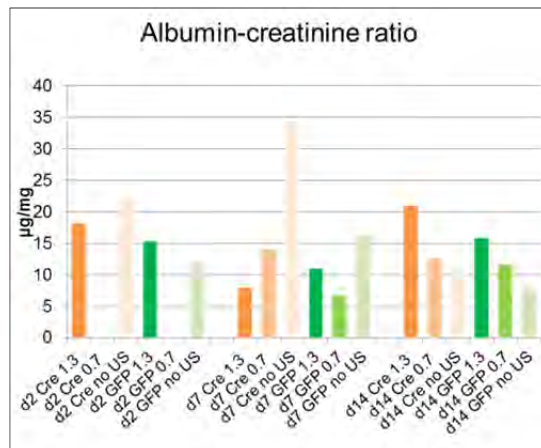


Figure 8: Analysis of renal damage from urinary markers in MB/US treated mice. 24 hr urines were collected from mice after treatment with various US strengths and analyzed by ELISA for KIM-1, a marker of acute kidney injury, and for urinary albumin and creatinine, expressed as the ACR or albumin/creatinine ration. US treatment did not increase detectable markers of renal damage.

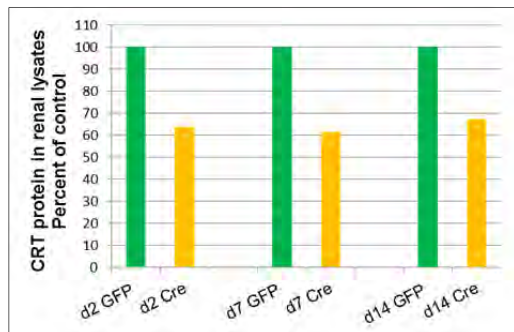
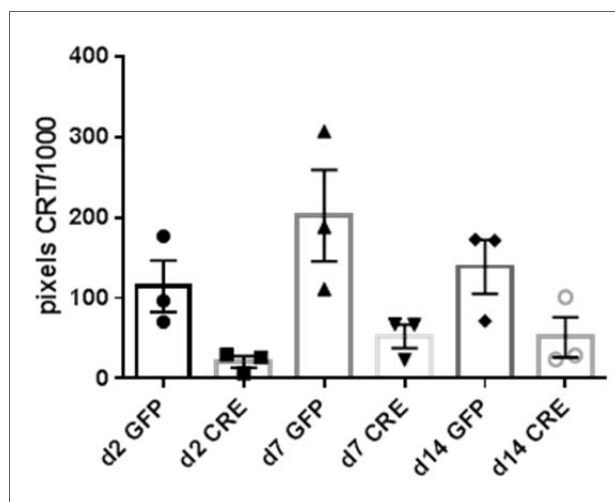
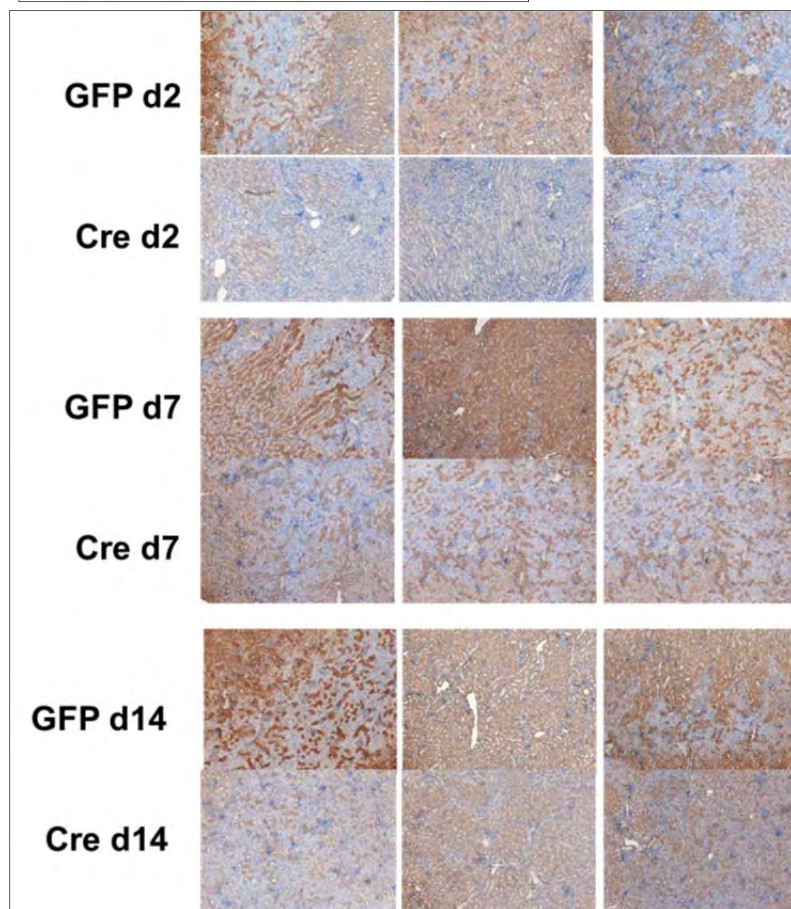


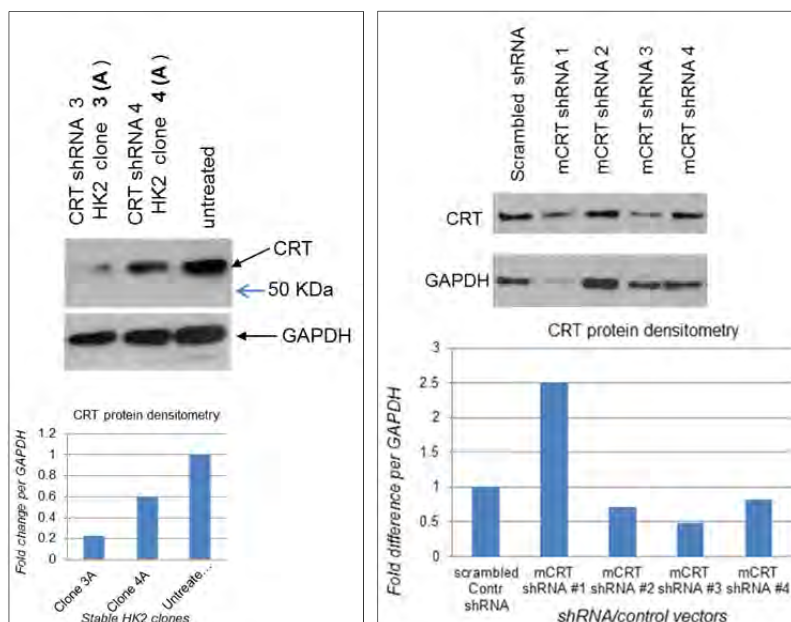
Figure 9. Delivery of Cre-recombinase plasmid (CRE) with microbubbles (MB) to CRT floxed mice via kidney-targeted Ultrasound (US) reduces CRT expression in kidneys. (A) CRT protein was reduced in renal lysates as detected by western blotting of renal tissue in mice receiving Cre-recombinase plasmid (CRE), but not in mice given control GFP plasmid. **(B)** Immunostaining for CRT using a rabbit monoclonal antibody to CRT on kidney sections from treated mice shows expression of CRT in renal tubules and reduced tubular CRT expression in mice receiving Cre-recombinase plasmid (original mag 10x). **(C)** Morphometric analysis of CRT immunostaining in renal sections from mice harvested at days 2, 7, and 14 following ultrasound treatment (n=3)/group, error bars are SEM.



Subtask 8: Induce diabetes with streptozotocin, uninephrectomy, delivery of plasmid through sonoporation, repeat as necessary, and monitor animals over 22 weeks (50 mice)

In March 2015, when we determined the feasibility of this approach, we scaled up breeding of the CRT floxed mice in order to have sufficient numbers of male mice to perform the diabetic nephropathy studies in the streptozotocin/uninephrectomy model. We now have sufficient male mice and will soon start these studies.

Figure 10. CRT shRNA reduces CRT expression in (A) HK-2 cells and (B) Mouse PTC cells.



Major Task 2 (Aim 2.b)

Subtask 1: identify active mouse CRT siRNA; develop shRNA plasmid under U6 promoter, and validate CRT knockdown with mouse cells (MEFS, mouse proximal tubule cells) in vitro.

We have identified 4 human CRT shRNA plasmids that reduce CRT expression in 293 HEK cells, transduced HK-2 cells and are selecting clones; showed reduced CRT in 2 separate HK-2 clones (**Figure 10 left**). We have also identified multiple shRNAs to knockdown mouse CRT (**Figure 10 right**) and

have packaged this shRNA into a lentivirus which will be used in the Akita model, and are in the process of selecting stable clones of transduced mouse PT cells. We are beginning work on lentivirus to deliver cre-recombinase and control GFP plasmids for use in the animal studies.

Summary

During year 1, we established that we can culture mouse proximal tubule cells, knockdown CRT, either via siRNA or shRNA, showed the kinetics of TGF-beta and glucose stimulation of FN and collagen I, and have preliminary data that CRT knockdown attenuates ECM production in response to TGF-beta stimulation. We also established the feasibility of renal knockdown of CRT in the pilot studies and established that markers of renal injury are not elevated by US/MB treatment. Although many of these in vitro studies will need to be repeated prior to publication, these initial data support our hypothesis and justify further in vitro studies as outlined in the proposal. We are now ready to proceed with the animal studies and expect to obtain results beginning in year 2.

c. WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

There was no specific intent to provide training or professional opportunities in this grant. However, Dr. Murphy-Ullrich has been providing direct mentoring to Dr. Borovjagin to assist him with learning cell biology techniques. He also attended the UAB Seminars in Nephrology weekly workshops in Spring 2015 to increase his background knowledge of renal disease.

d. HOW WERE THE RESULTS DISSEMINATED? *Nothing to report*

e. WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

During year 2, we will optimize the lentiviral-mediated knockdown of CRT in both the HK-2 and the mouse PT cells. We will complete the glucose studies with CRT shRNA knockdown in both HK-2 and mouse PTCs in the stably transduced cells. We will pursue Subtasks 2-4 in Major Task 2 (Specific Aim 1). We will also begin the calcium studies proposed in subtask 1 of major task 3.

In specific Aim 2, we will perform and complete the animal studies proposed in Major task 1 (subtasks 6-9) and Major task 2 (subtasks 1-5). We will also generate and validate the PEPCK promoter driven Cre-recombinase plasmid in the mouse PT cells and perform pilot studies in the CRT floxed mice.

4. IMPACT

- a. WHAT WAS THE IMPACT ON THE DEVELOPMENT OF THE PRINCIPAL DISCIPLINES OF THE PROJECT?** Nothing to report
- b. WHAT WAS THE IMPACT ON OTHER DISCIPLINES?** Nothing to report
- c. WHAT WAS THE IMPACT ON TECHNOLOGY TRANSFER?** Nothing to report
- d. WHAT WAS THE IMPACT ON SOCIETY BEYOND SCIENCE AND TECHNOLOGY?** Nothing to report

5. CHANGES/PROBLEMS

a. CHANGES IN APPROACH AND REASONS FOR CHANGE

In our pilot studies, we learned that most of the detectable CRT in the mouse kidney is in the tubules. Delivery of cre-recombinase via US/MB mediated delivery through the tail vein predominantly down-regulated CRT expression in the tubules (proximal, distal, and collecting ducts). Glomerular CRT under control and treated conditions was nearly undetectable. Therefore, we chose to delay the studies using the PEPCK- tubule specific

promoter to deliver the cre-recombinase plasmid to the proximal tubules and first proceed with non- cell targeted cre-recombinase plasmid. The PEPCK- promoter driven studies will follow the initial studies.

For in vitro studies, we had initial proposed using siRNA transient transfection via electroporation. Although this works for the HK-2 cells, transfection of the primary mouse proximal tubule cells was less efficient. We also saw that CRT knockdown via siRNA began to lose its effectiveness at 96 hrs. This shortened knockdown period could present a problem with some of the more delayed effects of glucose stimulation. To obtain more homogeneous populations with more stable knockdown, we switched to using a lentivirus expressing shRNA for CRT. The lentivirus production is going well with preliminary results showing several clones effective at knocking down CRT in HK-2 cells. We will be using this approach, and also generating lentivirus-Cre-recombinase viruses for use in the mouse PT cells. In the end, this will give us homogenous cells to use in our studies which will improve the reproducibility and significance of our data. The lentivirus GFP and Cre-recombinase will also be used for Aim 2.2 in the Akita mouse studies.

b. ACTUAL OR ANTICIPATED PROBLEMS OR DELAYS AND ACTIONS OR PLANS TO RESOLVE THEM

We had problems with efficiency of transfection of MPTEC with either GFP or Cre-recombinase plasmids, despite effort aimed at optimizing lipofectamine and electroporation approaches. In addition, CRT knockdown in the HK-2 cells by transient transfection of siRNA was only stable over a 72-96 hr time period. We are now using a lentivirus approach to increase transduction efficiency and to be able to select stably transduced cells which will give us a homogenous population of cells with stable knockdown of CRT. We initially have done this with lentiviral CRT shRNA for both human and mouse cell studies. We will also construct lentivirus expressing the cre-recombinase plasmid.

We were delayed in beginning the animal studies in Aim 2.1 (subtask 8,9) because of the time it took to breed 50+ male mice. These mice are now in hand and the studies will begin in the next few weeks.

c. CHANGES THAT HAD A SIGNIFICANT IMPACT ON EXPENDITURES

UAB did not grant raises in 2015 and the PI had a pay reduction. Therefore, personnel costs were less than originally budgeted. However, this will allow Dr. Murphy-Ullrich to increase her effort in year 2 (from 25 to 30%).

d. SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS, VERTEBRATE ANIMALS, BIOHAZARDS, AND/OR SELECT AGENTS

i. SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS not applicable

ii. SIGNIFICANT CHANGES IN USE OR CARE OF VERTEBRATE ANIMALS
SIGNIFICANT

We requested and were granted approval for a modification to our vertebrate animal use protocol. We will breed the CRT floxed mice (now on a mixed background) to C57Bl6 background. This will enable us to breed the floxed mice with renal specific Cre mice if we decide later in the project. Weiqi Lei, a lab technician was added to the protocol, although he is paid from other sources, as well as 92 mice for breeding purposes, and tail vein snips for phenotyping. UAB IACUC approval was obtained on 14-April 2015 and DoD/ACURO approval on 24 April 2015.

iii. CHANGES IN USE OF BIOHAZARDS AND/OR SELECT AGENTS none

6. PRODUCTS

a. PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS

i. JOURNAL PUBLICATIONS none

ii. BOOKS none

iii. OTHER PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS none

b. WEBSITE OR OTHER INTERNET SITES none

c. TECHNOLOGIES OR TECHNIQUES none

d. INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES none

e. OTHER PRODUCTS none

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. WHAT INDIVIDUALS WORKED ON THE PROJECT?

Name:	Joanne Murphy-Ullrich	Paul Sanders	Ailing Lu	Anton Borovjagin	Weqi Lei
Project Role:	PI	collaborator	Research associate	Research Associate	technician
Research Identifier	emurph (eCommons)	psanders (eCommons)	none	aborov (eCommons)	none
Person months worked	3.0	1.2	100	100	0
contribution	Oversight of the entire project, IACUC compliance, personnel supervision	Consultation on possible inducible Cre models specific for proximal tubule	Pilot animal studies and analyses (IHC, western blot), renal injury markers	Mouse and human cell culture, siRNA, lentivirus sRNA generation/validation; TGF-beta and glucose analysis	Supervise breeding and phenotyping of CRT-floxed mice to C57bl/ background (part of general lab responsibilities)
Funding support	NA	NA	NA	NA	NIH/NCI 1R01CA175012

b. HAS THERE BEEN A CHANGE IN THE ACTIVE OTHER SUPPORT OF THE PD/PI OR SENIOR/KEY PERSONNEL?

Murphy-Ullrich

(Grant support completed since 5/2014)

American Society for Hematology Bridge Grant (Murphy-Ullrich, PI)

03/01/14-02/28/15

0.24 mcal onths

The thrombospondin1-TGF- β axis in multiple myeloma

Goals: This is a bridge grant to support studies contained in NIH R01CA175012

Overlap: None

Contact: Awards Coordinator, Meley Gebremedhin, mgebremedhin@hematology.org, American Society of Hematology 2021 L Street NW, Suite 900, Washington, DC 20036 Phone 202-776-0544 | Fax 888-719-7814

UAB Comprehensive Cancer Center (Murphy-Ullrich PI)

12/01/13-11/30/14

0.48 cal months

The thrombospondin1-TGF- β axis in multiple myeloma

To investigate the mechanisms of TSP1 regulation of latent TGF- β activation on multiple myeloma progression in (Aim 1) an immune competent model of mouse myeloma and to (Aim 2) investigate TSP regulation of myeloma cell behaviors and bone cell interactions in vitro.

Overlap: None

Contact: Victor S Stark, Program Dir III, UAB, Comprehensive Cancer Center, WTI 260A, 2nd Avenue South, Birmingham, AL 35294-1720, vstark@uab.edu, 205-934-0022

IH/NIDDK P30 DK074038 (B. Yoder, PI)

10/01/10-06/30/15

0.60 cal months **UAB Hepatobiliary Fibrocystic**

Diseases Research and Translational Core Center

Dr. Murphy-Ullrich heads the pilot and feasibility grant program focused on mechanisms of fibrocystic diseases due to ciliopathies. She only receives salary support.

Overlap: None

Contact: Diana Ly and Dr. Michael Fleissner, National Institute of Diabetes and Digestive and Kidney Diseases/NIH/DHHS, Bldg 31, Rm 9A06, 31 Center Drive, MSC 2560, Bethesda, MD 20892-2560 USA 301.496.3583

New support

NIH/NCI (1R01CA175012-01) (Murphy-Ullrich, PI)

07/01/14-06/30/19

2.40 cal months

The Thrombospondin1-TGF-Beta Axis in Multiple Myeloma

Goals: (Aim 1) To determine the mechanism by which antagonism of TSP1-dependent TGF-beta activation impedes multiple myeloma growth and bone disease in immune competent models and in in vitro studies and (Aim 2) to develop stable peptide derivatives for therapeutics.

Overlap: None

Contact: Suzanne L. Forry, forryscs@mail.nih.gov, 240-276-5922 NIH, 9000 Rockville Pike, Bethesda MD, 20892

Eyesight Foundation of Alabama (38-2009-633) (Downs PI)

12/31/13-12/30/15

0.60 cal months

(Murphy-Ullrich, PI subproject)

Role of TSP1-TGF-beta in biomechanical remodeling in glaucoma

Goals: To examine the role of TSP1 control of cell adhesion and TGF-beta activation in sclera remodeling under differing biomechanical forces in models of glaucoma.

Overlap: None

Contact: Torrey V. A. DeKeyser, Executive Director, Shirley Hamilton, Director of Grants and Programs, Eyesight Foundation of Alabama Board of Trustees, 700 South 18th Street, Suite 123, Birmingham, AL 35233 205/488-0771 Phone 205/325-8335 Fax

Paul Sanders

(Grant support completed since 5/2014)

(1 IO1 BX001192 (PI: Sanders) 04/1/1986 - 03/31/2015 (current cycle years 25-28 of grant support)

Department of Veterans Affairs Merit Award Effort: 2.4 cal months *Low Molecular Weight Protein Nephrotoxicity*

The goal of this ongoing project is to determine the pathophysiology of light chain-related renal disorders that include cast nephropathy ("myeloma kidney") and proximal tubular epithelial cell injury.

Contact: Grants Management Specialist: Parvez, Zaheer 202-443-5702 Email: Zaheer.Parvez@VA.gov
U.S. Department of Veterans Affairs 810 Vermont Avenue, NW Washington, DC 20420

New Support

1 I01 CX001326 (PI: Sanders) 04/1/1986 - 03/31/2019 (current cycle years 29-33 of grant support)

Department of Veterans Affairs Merit Award

Effort: 2.4 calendar *Low Molecular Weight*

Protein Nephrotoxicity

The goal of this ongoing project is to determine the pathophysiology of light chain-related renal disorders that include cast nephropathy ("myeloma kidney") and proximal tubular epithelial cell injury.

c. WHAT OTHER ORGANIZATIONS WERE INVOLVED AS PARTNERS? None

8. SPECIAL REPORTING REQUIREMENTS Not applicable, None

9. APPENDICES none